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(54) Title: RESIDUAL PROTEASE-III		
(57) Abstract A <i>Bacillus</i> cell containing a mutation in the residual protease-III (rp-III) gene resulting in the inhibition of the production by the cell of proteolytically active RP-III.		

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Residual Protease-III

Background of the Invention

This invention relates to Bacillus strains useful for the expression and secretion of desired polypeptides (as used herein, "polypeptide" means any useful chain of amino acids, including proteins).

Bacillus strains have been used as hosts to express heterologous polypeptides from genetically engineered vectors. The use of a Gram positive host such as Bacillus avoids some of the problems associated with expressing heterologous genes in Gram negative organisms such as E. coli. For example, Gram negative organisms produce endotoxins which may be difficult to separate from a desired product. Furthermore, Gram negative organisms such as E. coli are not easily adapted for the secretion of foreign products, and the recovery of products sequestered within the cells is time consuming, tedious, and potentially problematic. In addition, Bacillus strains are non-pathogenic and are capable of secreting proteins by well-characterized mechanisms.

A general problem in using Bacillus host strains in expression systems is that they produce large amounts of proteases which can degrade heterologous polypeptides before they can be recovered from the culture media. The production of the majority of these proteases occurs at the end of the exponential growth phase. At this time, conditions of nutrient deprivation exist and the cells are preparing for sporulation. The two major extracellular proteases are an alkaline serine protease (subtilisin), the product of the apr gene, and a neutral metalloprotease, the product of the npr gene. Secretion of these proteases occurs into the medium, whereas the major intracellular

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serine protease, Isp-I, is produced within the cells. Other investigators have created genetically altered Bacillus strains that produce below normal levels of one or more of these three proteases. These strains still produce high enough levels of protease to cause the degradation of heterologous gene products prior to purification.

5 Stahl et al. (J. Bact., 1984, 158:411) disclose a Bacillus protease mutant in which the chromosomal subtilisin structural gene was replaced with an in vitro derived deletion mutation. Strains carrying this mutation had only 10% of the wild-type extracellular production of serine protease activity. Yang et al. (J. Bact., 1984, 160:15) disclose a Bacillus protease mutant in which the chromosomal neutral protease gene was replaced with a gene having an in vitro derived deletion mutation. Fahnestock et al. (WO 15 86/01825) describe the construction of Bacillus strains lacking subtilisin activity by replacing the native chromosomal gene sequence with a partially homologous DNA sequence containing an inserted inactivating segment.

10 Kawamura et al. (J. Bact., 1984, 160:442) disclose Bacillus strains carrying lesions in the npr and apr genes. These strains express less than 4% of the extracellular protease activity levels observed in wild-type strains. Koide et al. (J. Bact., 1986, 167:110) disclose the cloning and 20 sequencing of the isp-1 gene and the construction of an Isp-1 negative mutant by chromosomal integration of an artificially deleted gene.

25 Sloma et al., 1990 J. Bact. 172:1024-1029, employed B. subtilis deleted for the three major proteases (apr, npr, isp) in order to identify three additional residual proteases (epr, bpr, mpr). Blackburn et al., WO 89/10976 also used sporulation competent apr-, npr- strains to isolate what they alledge to be a residual serine protease

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(rsp) which lacks amino terminal homology to known Bacillus proteases.

Genetically altered strains which are deleted for both the major extracellular protease genes (apr and npr) and three residual protease genes (epr, bpr, mpr) produce virtually undetectable levels of protease activity in standard protease assays. However, a resorufin-labeled casein substrate, can be used to detect minor protease activities which are responsible for degradation of some heterologous polypeptides and proteins.

Summary of the Invention

The invention provides a novel protease, RP-III, and improved Bacillus cells containing mutations in the previously uncharacterized RP-III encoding gene (vpr); the cells also preferably contain mutations in the one or more or any combination of extracellular protease encoding apr, npr, epr, bpr, and mpr genes, resulting in the inhibition by the cells of production of these proteases. The bpr and mpr genes are also known as rp-I and rp-II, respectively.

Preferably, the mutation of the invention involves a mutation in the rp-III gene (recently named vpr) which inhibits the production by the cell of the proteolytically active RP-III. (As used herein, mutation can refer to a deletion within or of the coding region of a gene, a substitution of one or more base pairs for one or more naturally occurring base pairs, or an insertion of one or more base pairs within the coding region of a gene.) Most preferably, the mutation of the invention is a deletion within the coding region of the gene, including deletion of the entire coding region; alternatively, the mutation can consist of a substitution of one or more base pairs for naturally occurring base pairs, or an insertion within the protease coding region.

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The Bacillus cells of the invention may also contain a mutation in the isp-1 gene encoding intracellular serine protease I and may, in addition, contain a mutation which blocks sporulation and thus reduces the cell's capacity to 5 produce sporulation dependent proteases; preferably, this mutation blocks sporulation at an early stage, most preferably, this mutation is the spoOA mutation (described below). The invention further provides a method for producing stable heterologous polypeptides in a Bacillus 10 host cell by modifying the host to contain mutations in the apr, npr, and rp-III genes and in one or more of the genes including the epr gene, the bpr gene, and the mpr (rp-II) gene. The method may include introducing into the Bacillus host cell a gene encoding a heterologous polypeptide that is 15 modified so as to be expressed in the Bacillus host; such gene modifications may include but are not limited to a compatible promoter sequence, enhancer sequence, and/or ribosome binding site.

The invention also features purified DNA, expression 20 vectors containing DNA, and host Bacillus cells transformed with DNA encoding RP-III; preferably, such DNA is derived from Bacillus subtilis.

The invention also features the isolation of a substantially pure previously uncharacterized residual 25 protease (RP-III); as used herein, "substantially pure" means greater than 90% pure by weight.

The term "rp-III gene" herein means the respective gene corresponding to this designation in Bacillus subtilis, and the evolutionary homologues of this gene in other 30 Bacillus species, which homologues, as is the case for other Bacillus proteins, can be expected to vary in minor respects from species to species. In many cases, sequence homology between evolutionary homologues is great enough so that a

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gene derived from one species can be used as a hybridization probe to obtain the evolutionary homologue from another species, using standard techniques. In addition, of course, those terms also include genes in which base changes have
5 been made which, because of the redundancy of the genetic code, do not change the encoded amino acid residue or which produce conservative changes (to an amino acid of similar hydrophobicity or charge distribution) to a few amino acids.

Using the procedures described herein, we have
10 produced Bacillus strains which are significantly reduced in their ability to produce proteases, and are therefore useful as hosts for the expression, without significant degradation, of heterologous polypeptides capable of being secreted into the culture medium. We have found that the
15 Bacillus cells of the invention, even though containing several mutations in genes encoding related activities, are not only viable but healthy.

Any desired polypeptide can be expressed according to the invention, e.g., medically useful proteins such as
20 hormones, vaccines, antiviral proteins, antitumor proteins, antibodies or clotting proteins; and agriculturally and industrially useful proteins such as enzymes or pesticides, and any other polypeptide that is normally degraded by RP-III.

25 Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of Preferred Embodiments

The drawings will first be briefly described.

Drawings

Fig. 1 is a comparison of N-terminal sequence of RP-III to a composite N-terminal sequence deduced from known

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B. subtilis serine protease sequences encoded by apr, epr, bpr and isp-1.

Fig. 2 is the N-terminal sequence of RP-III and corresponding sequence of the "guess-mer" oligonucleotide 5 probe used to identify the rp-III gene.

Fig. 3 is a restriction map of a DNA fragment containing the rp-III coding region and shows approximate locations of rp-III subclones.

Fig. 4 is the DNA sequence of DNA encoding the rp- 10 III gene.

General Strategy for Creating Protease Deficient Bacillus Strains

General Methods

In order to detect residual protease activity 15 remaining in B. subtilis after removal of other known proteases, a strain must be constructed which lacks the known proteases. A Bacillus strain which is substantially devoid of extracellular proteolytic activity is described in EPA 0 369 817 A2, by Sloma et al., hereby incorporated by reference. A similar strain which contains multiple 20 mutations which inactivate apr, npr, isp-1, epr, bpr, and mpr was prepared and assayed for residual serine protease activity using resorufin-labeled casein (Boehringer-Mannheim) as a substrate. Residual serine protease RP-III 25 was detected in the multiply mutated strain; its activity was monitored throughout purification using the same substrate. The purification and characterization of RP-III and isolation of the gene encoding RP-III are described below, along with a procedure for generating a Bacillus 30 strain containing a mutation which inactivates the RP-III-encoding gene.

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General Methods

Construction of a multiply-mutated Bacillus strain is described by Sloma et al EPA 0 369 817 A2. Isolation of B. subtilis chromosomal DNA was as described by Dubnau et al., (1971, J. Mol. Biol., 56: 209). B. subtilis strains were grown on tryptose blood agar base (TBAB) (Difco Laboratories) or minimal glucose medium and were made competent by the procedure of Anagnostopoulos et al., (J. Bact., 1961, 81: 741). E. coli JM107 was grown and made competent by the procedure of Hanahan (J. Mol. Biol., 1983, 166: 587). Plasmid DNA from B. subtilis and E. coli were prepared by the lysis method of Birnboim et al. (Nucl. Acid. Res., 1979, 7: 1513). Plasmid DNA transformation in B. subtilis was performed as described by Gryczan et al., (J. Bact., 1978, 134: 138).

Protease assays

Resorufin-labelled casein or ¹⁴C-casein was used for RP-III assays. Culture supernatant samples were assayed either 2 or 20 hours into stationary phase. Inhibitors were pre-incubated with the supernatant for 30 minutes at room temperature. Where a very small amount of residual protease activity was to be measured, ¹⁴C-casein or resorufin-labelled casein was used as the substrate.

In the ¹⁴C-casein test, culture supernatant (100 µl) was added to 100 µl of 50mM Tris, 5mM CaCl₂, pH 8, containing 1 X 10⁵ cpm of ¹⁴C casein (New England Nuclear). The solutions were incubated at 37° C for 30 minutes. The reactions were then placed on ice and 20 µg of BSA were added as carrier protein. Cold 10% TCA (600 µl) was added and the mix was kept on ice for 10 minutes. The solutions were centrifuged to spin out the precipitated protein and the supernatants counted in a scintillation counter.

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The resorufin-labeled casein assay involved incubation of culture supernatant with an equal volume of resorufin-labelled casein in 50 mM Tris, 5mM CaCl₂, pH 8.0, at 45° C for 1 hour. Following incubation, unhydrolyzed substrate was precipitated with TCA and centrifuged. The supernatant (400ml) was made alkaline with 500mM Tris (pH 8.8) and the resulting chromogenic supernatant was quantitated spectrophotometrically at 574 nm.

Parental Strains

10 A number of Bacillus strains were used as sources for strains of the current invention.

Strain GP216, containing deletions within the four protease genes (apr, npr, isp-1, and epr), and strain GP240, containing deletions with the five protease genes (apr, npr, isp-1, epr, and bpr (rp-I)), were prepared as described by Sloma et al., EPA 0 369 817 A2. Strain GP241, isogenic to GP240 except for the hpr gene, was constructed from strain GP216 by transformation of GP216 with a plasmid (pUC derivative called pJMhpr2, Perego and Hoch, J. Bacteriology 170:2560, 1988) containing a mutated hpr gene and a cat gene. hpr encodes a repressor of protease production in Bacillus. GP216 was transformed with pJMhpr2 and transformants were selected on chloramphenicol. Chromosomal DNA was extracted from chloramphenicol resistant colonies and analyzed by Southern hybridization. One clone was recovered which contained two copies of the hpr-2 gene resulting from a double crossover between homologous sequences on the vector and in the chromosome. The clone was grown in the absence of drug selection, and one chloramphenicol sensitive colony was designated BI114. Strain GP241 was constructed by introducing the deleted bpr (rp-I) gene into BI114 using the plasmid pKT3 in the same

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manner as described in Sloma et al. (EPA 0 369 817 A2) for the introduction of the deleted bpr (rp-I) gene into GP216 generating GP240.

Strain GP263, carrying a mutation in mpk was prepared from GP241 as follows. Plasmid pCR125, carrying the phleomycin resistance gene inserted in a deleted mpk gene (Sloma et al., EPA 0 369 817 A2), was digested with EcoR1 and the linear plasmid DNA was used to transform GP241 to phleomycin resistance. Resistant transformants were selected by plating the transformed cells onto TBAB plates containing a gradient of 0-5 µg/ml phleomycin across the plate. Transformants that were resistant to approximately 2.5 ug/ml phleomycin on the plates were single colony purified on TBAB phleomycin plates and thereafter grown on TBAB without selective antibiotic. One transformant isolated following this treatment was designated GP263.

GP263 was used to generate two additional strains, GP264 and GP275. GP264 has the sacQ* regulatory element chromosomally integrated via transformation with the plasmid pDP104, as described by Sloma et al., EPA 86308356.4. GP275 was produced by fully deleting the already-inactivated mpk (rp-II) gene from GP263. Since inactivation of mpk was due to an insertion of the phleomycin resistance gene into mpk, the deletion of mpk was accomplished by transformation of GP263 with a plasmid containing a deleted mpk and chloramphenicol resistance genes in contiguous array. Transformants were selected on chloramphenicol. Isolated colonies were then grown in the absence of selection and replica plated. GP275 was isolated as both chloramphenicol and phleomycin sensitive.

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Identification of A Novel Proteolytic Activity

Extracellular protease levels are reduced in culture supernatants of Bacillus strains that do not express the proteases encoded by the six non-essential protease genes,
5 apr, npr, isp-1 epr, bpr and mpr. When these deletions are present in a Sp⁺ host, there is an approximate 99% reduction in extracellular protease levels compared to the wild-type strain. In order to efficiently produce protease labile products in Bacillus, it is desirable to decrease or
10 eliminate the remaining 1% residual protease activity.

Using the resorufin-labeled casein assay, a novel protease has been identified which is a major component of the residual activity in GP264. This protease may be classified as a serine protease by virtue of its
15 quantitative inhibition by phenylmethylsulfonyl fluoride.

Isolation and Characterization of RP-III

A simple and efficient purification scheme was developed for the isolation of the RP-III protease from spent culture fluids. Cultures were grown in modified MRS
20 lactobacillus media (Difco, with maltose substituted for glucose) and concentrated approximately 20-fold using an Amicon CH2PR system equipped with a S1Y10 spiral cartridge and dialyzed in place against 50mM MES pH 5.5, and allowed to incubate overnight at 0-4°C. The concentrated, crude
25 supernatant containing precipitated protein was centrifuged (Sorvall GSA rotor, 9000 rpm, 30 minutes) and the resulting pellet containing 80-100% of the RP-III protease activity was resuspended in 100 mM Tris, pH 8. The reconstituted pellet was then applied to a 500 ml Superflo (Sepragen)
30 column packed with Q-Sepharose (Pharmacia) equilibrated with 100mM Tris, pH 8. Bound protein containing the RP-III protease was recovered from the column with a 50mM MES, 2.5 M KCl, pH 5.5, step elution.

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The high-salt fractions containing protease activity were pooled, concentrated and dialyzed against 50mM MOPS, pH 7, then applied to a 250 ml Superflo column of benzamidine Sepharose (Pharmacia) affinity resin equilibrated with the same buffer. Bound RP-III protease was eluted from the resin with a step of 50mM MOPS, 1 M KCl, pH 7.

Proteolytically active high-salt fractions containing RP-III protease were pooled, concentrated and subjected to HPLC size-exclusion chromatography over a semi-preparative SW3000 column equilibrated with 50mM MES, 200mM KCl, pH 6.8.

Protease activity was found exclusively in the void volume indicating the RP-III protease exists as part of a large aggregate. Finally, the size-excluded RP-III pool was concentrated, dialyzed against 20mM sodium phosphate, 1M NaCl, 1mM imidazole, pH 7.5, and fractionated over a Progel-TSK chelate-5PW HPLC column charged with Cu⁺⁺. Activity was eluted with a linear gradient of imidazole to 20mM.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed that the final pool of RP-III protease contained three major Coomassie-staining bands: one at 38.4 kDa and a doublet at 28.5 and 27.1 kDa. Each of these bands were electrophoretically transferred to and cut out of a sheet of PVDF membrane and subjected to amino-terminal sequence analysis. The sequence of the 28.5 kDa protein bore remarkable homology (81%) to a composite sequence of four other *B. subtilis* serine proteases (*apr*, *subtilisin*; *epr*, extracellular protease; *bpr*, Bacillopeptidase F, and *isp-1*, intracellular protease 1) as well as to Bacillopeptidase F itself (65% homology). The proteolytic activity in this band is referred to herein as RP-III. Figure 1 illustrates the amino-terminal sequence of RP-III and its comparison to a composite sequence deduced

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from the amino acid sequences of the aformentioned B.
subtilis serine proteases.

All five proteases contain six identical residues
spaced exactly the same within the N-termini, including the
5 putative active center aspartic acid residue.

Sequence analysis of the 27.1kDa lower band revealed it is
most likely a proteolytic fragment of the 28.4kDa upper band
since both proteins have identical amino-terminal sequences
from residue 10 to residue 29. The loss of residues 1-9 on
10 the lower 27.1kDa band accounts for its faster mobility on
SDS-PAGE compared to the upper 28.4kDa band.

Figure 2 shows the amino-terminal sequence obtained
from RP-III and the sequence of the oligomeric probe
constructed to identify the gene that codes for RP-III.

15 Cloning and Sequencing of the rp-III Gene.

Genomic DNA was prepared from Bacillus subtilis
GP275, and 10 µg were exhaustively digested with HindIII and
probed with the guess-mer shown in Fig 2. The probe
hybridized to a 1kb fragment of HindIII-digested genomic
20 DNA; therefore, a 1kb genomic library was prepared from
size-selected fragments of 0.8-1.5 kb, using pUC19 as the
vector. A clone carrying the rp-III gene was identified in
the 1 kb library using standard hybridization techniques
(Sambrook et al., 1989, Molecular Cloning, Cold Spring
25 Harbor, NY) and the guess-mer probe shown in Fig. 2. The
plasmid isolated from this clone was designated pLLP1.

Southern blot analysis was used to determine the
location of useful restriction sites with the rp-III gene
(Fig. 3). Southern blots were performed using restriction
30 digests of genomic DNA from GP275 and a probe encompassing
the 1kb HindIII fragment from pLLP1. These results led to
the preparation of size-selected EcoR1, EcoR1/BglII,
EcoR1/HindIII and BglII libraries from GP275 genomic DNA.

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Libraries yielding useful clones were prepared in either pIC20H or in pUC19 vectors digested with the appropriate restriction enzymes. pLLP4 and pLLP5 were isolated from 3kb and 0.5-0.8kb EcoR1/BglII pIC20H libraries, respectively, by 5 screening with the 1kb HindIII fragment of pLLP1. pLLP8 was isolated from a 0.5-0.8kb EcoR1/HindIII pUC19 library by screening with the 630 bp BglII fragment of pLLP5.

These clones were used to construct a restriction map of the rp-III gene, after the regions flanking the 1kb HindIII fragment were identified. The DNA sequence was determined between the 5' BglII site of pLLP5 and approximately 1kb beyond the 3' HindIII site of pLLP4 (Figs. 3 and 4).

An open reading frame was found to extend 2457 15 nucleotides downstream from the 5' BglII site. A putative translation initiation codon was identified (Fig 4, underlined nucleotides 40-42), with an accompanying ribosome binding site (Fig. 4, underlined nucleotides 25-32). The amino terminal sequence of the mature protein corresponding 20 to the sequence in Figure 2, was found at nucleotide 520 and is underlined in Figure 4. From the sequence data of Figure 4, the mature protein encoded by the rp-III gene is expected to contain 646 amino acids. Since the isolated protein has an apparent molecular weight of 28,000 d., this would 25 suggest that rp-III undergoes extensive C-terminal processing or proteolysis.

Location of the rp-III Gene on the
B. Subtilis Chromosome

Identification of the chromosomal location of the 30 rp-III gene may be accomplished by standard methods, essentially as described by Sloma et al. EPA 0 369 817 A2, for other protease genes. Briefly, the location of the rp-III gene on the B. subtilis chromosome was mapped by

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integrating a drug resistance marker into the chromosome at
the site of rp-III and using phage PBS1-mediated
transduction to determine the location of the drug
resistance gene. A fragment containing a neomycin
5 resistance (neo) gene was cloned into the BglII site within
the amino terminal coding region of rp-III, as described
below to give plasmid pLLP2 which was used to create GP279.
Southern blotting techniques and hybridization were used to
confirm that the neo gene had integrated into the
10 chromosome, interrupting the rp-III gene. Mapping
experiments were then used to indicate that the inserted neo
gene and rp-III are linked to the known Bacillus genetic
locations, sacA, ctr, and epr, by PBS1 transduction.

Inactivation of the rp-III gene

15 It is often useful to inactivate the production of
functional RP-III protease in microorganisms, particularly
when a desired protein is being produced which is
susceptible to RP-III proteolysis. The rp-III gene sequence
provided herein allows for elimination of RP-III activity by
20 any number of standard methods; including inactivation by
insertion of nucleotide sequences into the gene, or by
deletion of part or all of the native gene. In general,
homologous recombinant techniques may be employed; for
example, see Sloma et al. EPA 0 369 817 A2.

25 The rp-III gene was inactivated by creating an
insertion mutation within the native gene. A 2.4kb SmaI to
SmaI fragment containing the entire neomycin resistance gene
was inserted into the Klenow blunt-ended BglII site of
pLLP1, to give the plasmid pLLP2. pLLP2 was then linearized
30 by ScaI digestion and used to transform Bacillus strain
GP275. Neomycin resistant strains from this transformation
were called GP279 and contained an inactivated rp-III gene.
The inactivation of rp-III was confirmed by protease

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activity assay, as described above. Strains bearing the insertion mutation were otherwise normal with regard to sporulation and growth.

Heterologous DNA Expression

5 Cells in which the rp-III gene has been inactivated may be employed to express useful heterologous proteins. Such proteins would typically be of medical, agricultural, or industrial significance. In order to minimize any potential proteolytic damage of the heterologous protein, 10 preferred cells will also be inactivated for apr, npr, epr, bpr, and mpn. Inactivation of additional genes such as isp-1 and spooA may also be useful.

DNA encoding the desired heterologous proteins must be engineered to contain the proper regulatory sequences 15 including promoter, ribosome binding site, and transcription termination signals. In general, the DNA sequence encoding the protein and its accompanying regulatory sequences must be compatible with expression in the Bacillus host cell of the invention. The introduced DNA containing the expression 20 sequences may reside within the cell in plasmid form or more preferably it may be chromosomally integrated.

The following references are incorporated herein by reference: Guidelines and references for heterologous protein expression and selection of appropriate Bacillus regulatory elements are given in Ganesan et al., 1986 Bacillus Molecular Genetics and Biotechnology Applications. Academic press pp. 367-493. Methods useful for the construction of expression vectors are given by Sambrook et al., 1989, Molecular Cloning a Laboratory Manual Cold Spring 30 Harbor Laboratory Press.

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Other Embodiments

Other embodiments are within the following claims. For example, in some instances it may be desirable to express, rather than mutate or delete, the gene encoding RP-III; for example, to produce the protease for purposes such as improvement of the cleaning activity of laundry detergents or for use in industrial processes. This can be accomplished either by inserting regulatory DNA (any appropriate Bacillus promoter and, if desired, ribosome binding site and/or signal encoding sequence) upstream of the protease-encoding gene or, alternatively, by inserting the protease-encoding gene into a Bacillus expression or secretion vector; the vector can then be transformed into a Bacillus strain for production (or secretion) of the protease, which is then isolated by conventional techniques. Alternatively, the protease can be overproduced by inserting one or more copies of the protease gene on a vector into a host strain containing a regulatory gene such as sacQ*.

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Claims

1. A Bacillus cell containing a mutation in the rp-III gene resulting in inhibition of the production by said cell of proteolytically active RP-III.

5

2. The Bacillus cell of claim 1, further comprising a mutation in each of one or more protease-encoding genes selected from the group: apr, npr, epr, bpx, and MPr, wherein each said mutation results in inhibition of the production by said cell of proteolytically active protease encoded by said gene.

10

3. The Bacillus cell of claim 2, each said mutation comprising a deletion within the coding region of said gene.

15

4. The Bacillus cell of claim 3, said cell further containing a mutation in the isp-1 gene encoding an intracellular protease.

20

5. The Bacillus cell of any of claims 1-4, said cell further containing a mutation which reduces said cell's capacity to produce one or more sporulation-dependent proteases.

25

6. The Bacillus cell of claim 5 wherein said sporulation-dependent protease mutation blocks sporulation at an early stage.

30

7. The Bacillus cell of claim 6, said sporulation-blocking mutation being in the spoOA gene.

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8. The Bacillus cell of claim 7, said cell being
Bacillus subtilis.

5 9. The Bacillus cell of any one of claims 1-4 and
6-8, further comprising a gene encoding a heterologous
polypeptide.

10 10. The Bacillus cell of claim 5 further comprising
a gene encoding a heterologous polypeptide.

15 12. The cell of claim 9 wherein said heterologous
polypeptide is a medically, agriculturally or industrially
useful protein.

20 16. A method for producing a heterologous
polypeptide in a Bacillus cell, said method comprising
introducing into said cell a gene encoding said heterologous
polypeptide, modified to be expressed in said cell, said
Bacillus cell containing mutations in the rp-III, apr and
npr genes.

25 17. The method of claim 16 wherein said cell
further contains mutations in one or more of the genes, epr,
bpr, or mpr.

30 18. The method of claim 17, said cell further
containing a mutation in the isp-1 gene encoding
intracellular protease I.

35 19. The method of claim 16, 17, or 18 wherein said
cell further contains a mutation which reduces said cell's
capacity to produce one or more sporulation-dependent
proteases, said mutation being in the spoOA gene.

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20. The method of claim 19 wherein said cell is a
Bacillus subtilis cell.

5 21. The method of claim 20 wherein said
heterologous polypeptide is a medically, agriculturally or
industrially useful protein.

10 22. Purified DNA comprising
a Bacillus rp-III gene.

15 23. A vector comprising a Bacillus rp-III gene and
regulatory DNA operationally associated with said gene.

24. A Bacillus cell transformed with the vector of
claim 23.

25. Substantially pure Bacillus RP-III protease.

20 26. The DNA of claim 22 wherein said sequence is
sequence ID No. _____ (Fig. 4).

FIG. 1 - N-TERMINAL AMINO ACID HOMOLOGY
BETWEEN RP-III AND OTHER B.
SUBTILIS SERINE PROTEASES (I.E.,
BPR, EPR, APR, ISP-I)

5

RP-III I G A N D A W D L G Y T G K G I K V A I I D T G V E
COMPOSITE I - A - - A W - L G Y T G K G I K V A - I D T G V E

10

△
△
ACTIVE CENTER ASP

COMPOSITE HOMOLOGY - 81%
BPR HOMOLOGY - 65%

15

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FIG. 2 -

AMINO-TERMINAL SEQUENCE OF RP-III AND
CORRESPONDING "GUESS-MER" PROBE SEQUENCE

1 2 3 4 5 6 7 8

H₃N -MET- ASP- ASP- SER- ALA- PRO- TYR- ILE-

5 5' -ATG GAT- GAT- TCT- GCA- CCG- TAT- ATT-

9 10 11 12 13 14 15 16

GLY- ALA- ASN- ASP- ALA- TRP- ASP- LEU-

GGA- GCA- AAT- GAT- GCA- TGG- GAT- CTT-

10

17 18 19 20 21 22 23 24

GLY- TYR- THR- GLY- LYS- GLY- ILE- LYS-

GGA- TAT- ACA- GGA- AAA- GGA- ATT- AAA-

15

25 26 27 28 29 30 31 32

VAL- ALA- ILE- ILE- ASP- THR- GLY- VAL-

GTT-

33 34 35

20

GLU- TYR- ASN-

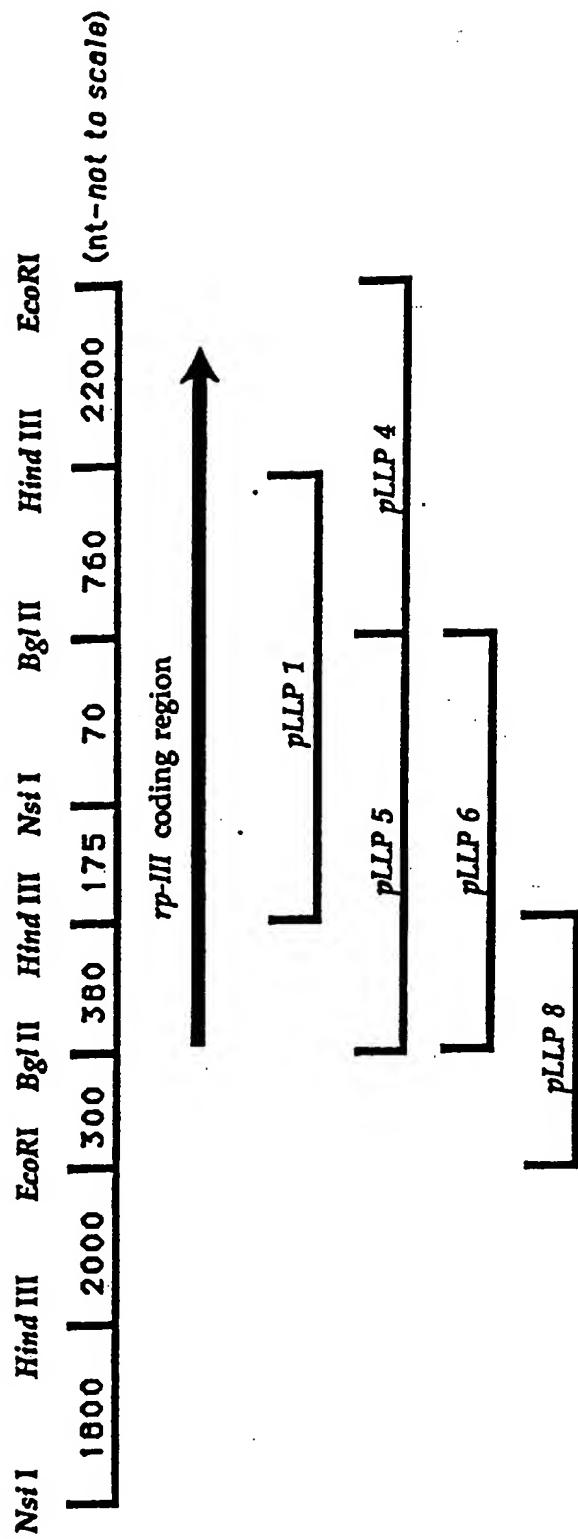


Figure 3

001 ATC TTT CAC RTT TTT TCT RAA TAC AAA GGG GGA, RAC RCA TTG RAA ARG GGG ATC ATT CGC
arg lys lys gly Ile Ile arg

061 TTT CTG CTT GTC AGT TTC GTC TTA TTT GCG TTA TCC ACA GGC ATT ACG GGC GTT CRG
 phe leu leu val ser phe val leu phe phe ala leu ser thr gly Ile thr gly val gln

121 GCA GCT CCG GCT TCT TCA RAA ACG TCG GCT GAT CTG GAA RAA GCC GAG GTC TTC GGT GAT
 ala ala pro ala ser ser lys thr ser ala asp leu glu lys ala glu val phe gly asp

181 ATC GAT ATG ACG RAC AGC RAA RAA RAC ACC GTT ATA GTG GAA TTA RAA GAA RAA TCC TTG
 Ile asp met thr thr ser lys lys thr thr val Ile val glu leu lys glu lys ser leu

241 GCA GAA GCG RAG GAA GCG GGA GAA RGC CRA TCG RAA RGC RAG CTG RAA ACC GCT CGC ACC
 ala glu ala lys glu ala gly glu ser gln ser lys ser lys leu lys thr ala arg thr

301 RAA GCA RAA RAC RAA GCA ATC RAA GCA GTG RAA RAC GCA RAA GTC RAC CGG GAA TAT GAG
 lys ala lys asn lys ala Ile lys ala val lys asn gly lys val asn arg glu tyr glu

361 CRG GTC TTC TCA GGC TTC TCT ATG AAG CTT CCA GCT RAT GAG ATT CCA RAA CTT CTA CGC
 gln val phe ser gly phe ser met lys leu pro ala asn glu Ile pro lys leu leu ala

421 GTC RAA GAC GTT RAG GCA GTG TAC CCG RAC GTC ACA TAT RAA ACA GAC RAT ATG RAG GAT
 val lys asp val lys ala val tyr pro asn val thr tyr lys thr asp asn met lys asp

481 RAA GRC GTC ACA ATC TCC GAA GRC GCC GTC TCT CCG CRA ATG GAT GRC AGT GCG CCT TAT
 lys asp val thr Ile ser glu asp ala val ser pro gln met asp asp ser ala pro tyr

541 ATC GGA GCA RAC GAT GCA TGG GAT TTA GGC TAC ACA GGA RAA GGC ATC RAG GTG GCG ATT
Ile glu ala asn asp ala trp asp leu gly tyr thr gly lys glu Ile Ius val ala Ile

601 ATT GAC ACT GGG GTT GAA TAC RAC CRA CCA GAT CTG RAG RAA RAC TTT GGA CRA TAT RAA
Ile asp thr gly val glu tyr asp his pro asp leu lys asn phe gly gin tyr lys

661 GGA TAC GAT TTT GTG GAC RAT GAT TAC GAT CCA RAA GAA RAC CCA ACC GGC GAT CGG AGG
 gly tyr asp phe val asp asn asp tyr asp pro lys glu thr pro thr gly asp pro arg

721 GGC GAG GCA ACT GAC CAT GGC ACA CRA GTC GCC GGA ACT GTG GCT GCA RAC GGA RCG ATT
 gly glu ala thr asp his gly thr his val ala gly thr val ala ala asn gly thr Ile

781 RAA GGC GTC GCG CCT GAT GCC ACA CTT CTT GCT TAT CGT GTG TTA GGG CCT GGC GCA RGC
 lys gly val ala pro asp ala thr leu leu ala tyr arg val leu gly pro gly gly ser

841 GGC ACA RCG GAA RAC GTC ATC GCG GGC GTG GAA CGT GCA GTG CRG GAC GGG GCA GAT GTG
 gly thr thr glu asn val Ile ala gly val glu arg ala val gln asp gly ala esp val

901 ATG RAC CTG TCT CTC GGA RAC TCT TTA RAC RAC CCG GAC TGG GCG RCA AGC RAC GCG CTT
 met asn leu ser leu gly asn ser leu asn asn pro asp trp ala thr ser thr ala leu

961 GAC TGG GCC ATG TCA GAA GGC GTT GTC GCT GTT ACC TCA RAC GGC RAC AGC GGA CGG RAC
 asp trp ala met ser glu gly val val ala val thr ser asn gly asn ser gly pro asn

1021 GGC TGG RAC GTC GGA TCG CCG GGC RAC TCA RGR GAA GCG ATT TCT GTC GGT GCG ACT CRG
 gly trp thr val gly ser pro gly thr ser arg glu ala Ile ser val gly ala thr gln

1081 CTG CCG CTC RAA GAA TAC GCC GTC ACT TTC GGC TCC TAC TCT TCA GCA RAA GTG ATG GGC
 leu pro leu asn glu tyr ala val thr phe gly ser tyr ser ser ala lys val met gly

1111 TAC AAC AAA GAG GRC GRC GTC RAA GCG CTC RAT RAC AAA GAA GTT GAG CTT GTC GAA GCG
tyr asn lys glu asp asp val lys ala leu asn asn lys glu val glu leu val glu ala
1201 GGA ATC GGC GAA GCA RAG GAT TTT GAA GGG RAA GAC CTG ACA GGC RAA GTC GGC GTT GTC
gly Ile gly glu ala lys asp phe glu gly lys asp leu thr gly lys val ala val val
1261 RAA CGA GGC AGC ATT GCA TTT GTG GAT RAA GCG GAT RAC GCT RAA RAA GCC GGT GCA ATC
lys arg gly ser Ile ala phe val asp lys ala asp asn ala lys ala gly ala Ile
1321 GGC ATG GTT GTG TAT RAC RAC CTC TCT GGA GAA ATT GAA GCC RAT GTG CCA GGC ATG TCT
gly met val val tyr asn asn leu ser gly glu Ile glu ala asn val pro gly met ser
1381 GTC CCA ACG ATT RAG CTT TCA TTA GAA GAC GGC GAA RAA CTC GTC AGC GCC CTG RAA GCT
val pro thr Ile lys leu ser leu glu asp gly glu lys leu val ser ala leu lys ala
1441 GGT GAG ACA RAA ACA RAA TTC RAG TTG ACG GTC TCA RAA GCG CTC GGT GAA CAA GTC GCT
gly glu thr lys thr thr phe lys leu thr val ser lys ala leu gly glu gln val ala
1501 GAT TTC TCA TCA CGC GGC CCT GTT ATG GAT ACG TGG ATG ATT RAG CCT GAT ATT TCC GCG
asp phe ser ser arg gly pro val met asp thr trp met Ile lys pro asp Ile ser ala
1561 CCA GGG GTC RAA ATC GTG AGC ACG RTC CCA ACA CRC GAT CCT GAC CAT CCA TAC GGC TAC
pro gly val asn Ile val ser thr Ile pro thr his asp pro asp his pro tyr gly tyr
1621 GGA TCA RAA CRA GGA ACA AGC ATG GCA TCG CCT CAT ATT GCC GGA GCG GTT GCC GTT ATT
gly ser lys gln gly thr ser met ala ser pro his Ile ala gly ala val ala val Ile
1681 RAA CRA GCC RAA CCA RAG TGG AGC GTT GAA CAG ATT RAA GCC GCC RTC ATG RAA RCC GCT
lys gln ala lys pro lys trp ser val glu gln Ile lys ala ala met asn thr ala
1741 GTC ACT TTA RAG GAT AGC GAT GGG GAA GAA GTC TAT CCG CAT RAC GCT CRA GGC GCA GGC AGC
val thr leu lys asp ser asp gly glu val tyr pro his asn ala gln gly ala gly ser
1801 GCA AGA ATT ATG RAC GCA RTC RAA GCC GAT TCG CTC GTC TCA CCT GGA AGC TAT TCA TAC
ala arg Ile met asn ala Ile lys ala asp ser leu val ser pro gly ser tyr ser tyr
1861 GGC ACG TTC TTG RAG GAA RAC GGA RAC GAA RAA RAA RAT GAA AGC TTT ACG ATT GAA RAA RAA
gly thr phe leu lys glu asn gly asn glu thr lys asn glu thr phe thr Ile glu asn
1921 CRA TCT TCC ATT AGA RAG TCA TAC ACA CTT GAA TAC TCA TTT RAT GGC AGC GGC ATT TCC
gln ser ser Ile arg lys ser tyr thr leu glu tyr ser phe asn gly ser gly Ile ser
1981 ACA TCC GGC ACA AGC CGT GTT GTG ATT CCG GCA CAT CRA ACC GGC RAA GTC ACT GCA RAA
thr ser gly thr ser arg val val Ile pro ala his gln thr gly lys ala thr ala lys
2041 GTC RAG GTC RAA AGC RAA RAA GCT GGC ACC TAT GAA GGA AGC GTT ATC GTC AGC
val lys val asn thr lys lys thr lys ala gly thr tyr glu gly thr val Ile val arg
2101 GAA GGC GGA RAA AGC GTC GCT RAG GTC CCT ACA TTG CTG ATT GTG RAA GAG CCC GAT TAT
glu gly gly lys thr val ala lys val pro thr leu leu Ile val lys glu pro asp tyr
2161 CCG AGA GTC ACA TCT GTC TCT GTC AGC GAA GGG TCT GTC CRA GGT ACC TAT CRA ATT GAA
pro arg val thr ser val ser glu gly ser val gln gly thr tyr gln Ile glu
2221 ACC TAC CTT CCT GCG GGA GCG GAA GAG CTG GCG TTC CTC GTC TAT GAC AGC AAC CTT GAT
thr tyr leu pro ala gly ala glu glu leu ala phe leu val tyr asp ser asn leu asp

2281 TTC GCA GGC CAA GCC GGC ATT TAT AAA AAC CAA GAT RRA GGT TAC CAG TAC TTT GAC TGG
phe ala gly gln ala gly ile tyr lys asn gln asp lys gly tyr gln tyr phe asp trp

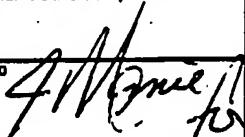
2341 GAC GGC ACG ATT AAT GGC GGA ACC AAA CTT CCG GCC GGA GAG TAT TAC TTG CTC GCA TAT
asp gly thr ile asn gly gly thr lys leu pro ala gly glu tyr tyr leu leu ala tyr

2401 GCC GCG ACG AAA GGC AAG TCA AGC CAG GTT TTG ACC GAA GAA CCT TTC ACT GTT GAA TAA
ala ala asn lys lys ser ser gln val leu thr glu glu pro phe thr val glu DCH

2461 GAAAAAGCCCTGCCGATTGGCAGGGCTTTTAAAGATCAGTCAGCARRCGCCTCCTGCATTAAGCGATACG

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/01598

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC (5) : C12P 21/02 US CL : 435/69.1, 219		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	435/69.1, 219	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁵		
BIOSIS, MEDLINE, WPI, APS, JPABS, EMBL, GENE BANK, UMBEL SEARCH TERMS; PROTEASE, REDUCED, BACILLUS, FIGURE 4		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category ⁶	Citation of Document, ¹⁵ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
Y	US, A, 4,828,994 (FAHNESTOCK ET AL) 09 MAY 1989, SEE ENTIRE DOCUMENT	1-21
Y	Journal of Bacteriology, Volume 160, No.1, issued October 1984, M. Y. Yang et al, "Cloning of the Neutral Protease Gene of <u>Bacillus subtilis</u> and the Use of the Cloned Gene to Create an In Vitro-Derived Deletion Mutation", pages 15-21, see entire document.	1-26
Y	EP, A, 0,257,189 (UDAKA ET AL) 02 MARCH 1988, SEE ENTIRE DOCUMENT.	1-21
Y	EP, A, 0,369,817 (SLOMA ET AL) 23 MARCH 1990, SEE ENTIRE DOCUMENT.	1-21
Y	WO, A, 86/01825 (FAHNESTOCK ET AL) 27 MARCH 1986, SEE ENTIRE DOCUMENT.	1-21
Y	US, A, 4,946,789 (UDEKA ET AL) 07 AUGUST 1990, SEE ENTIRE DOCUMENT.	1-21
Y	JOURNAL OF BACTERIOLOGY, VOLUME 158, NO.2, ISSUED MAY 1984, M. L. STAHL ET AL, "REPLACEMENT OF THE <u>BACILLUS SUBTILLISIN</u> STRUCTURAL GENE WITH AN IN VITRO-DERIVED DELETION MUTATION", PAGES 411-418, SEE ENTIRE DOCUMENT.	1-26
<p>* Special categories of cited documents:¹⁶</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ² 14 JUNE 1992	Date of Mailing of this International Search Report ² 29 JUN 1992	
International Searching Authority ¹ ISA/US	Signature of Authorized Officer ²⁰ DAVID B. SCHMICKEL 	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	EP, A, C.227,260 (SLOMA ET AL) 01 JULY 1987, SEE ENTIRE DOCUMENT.	1-26
Y	JOURNAL OF BACTERIOLOGY, VOLUME 172, NO. 2, ISSUED FEBRUARY 1990, A. SLOMA ET AL., "GENE ENCODING A NOVEL EXTRACELLULAR METALLOPROTEASE IN <u>BACILLUS SUBTILIS</u> ", PAGES 1024-1029, SEE ENTIRE DOCUMENT.	1-26

V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

1. Claim numbers , because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. Claim numbers , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

The additional search fees were accompanied by applicant's protest.
 No protest accompanied the payment of additional search fees.

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